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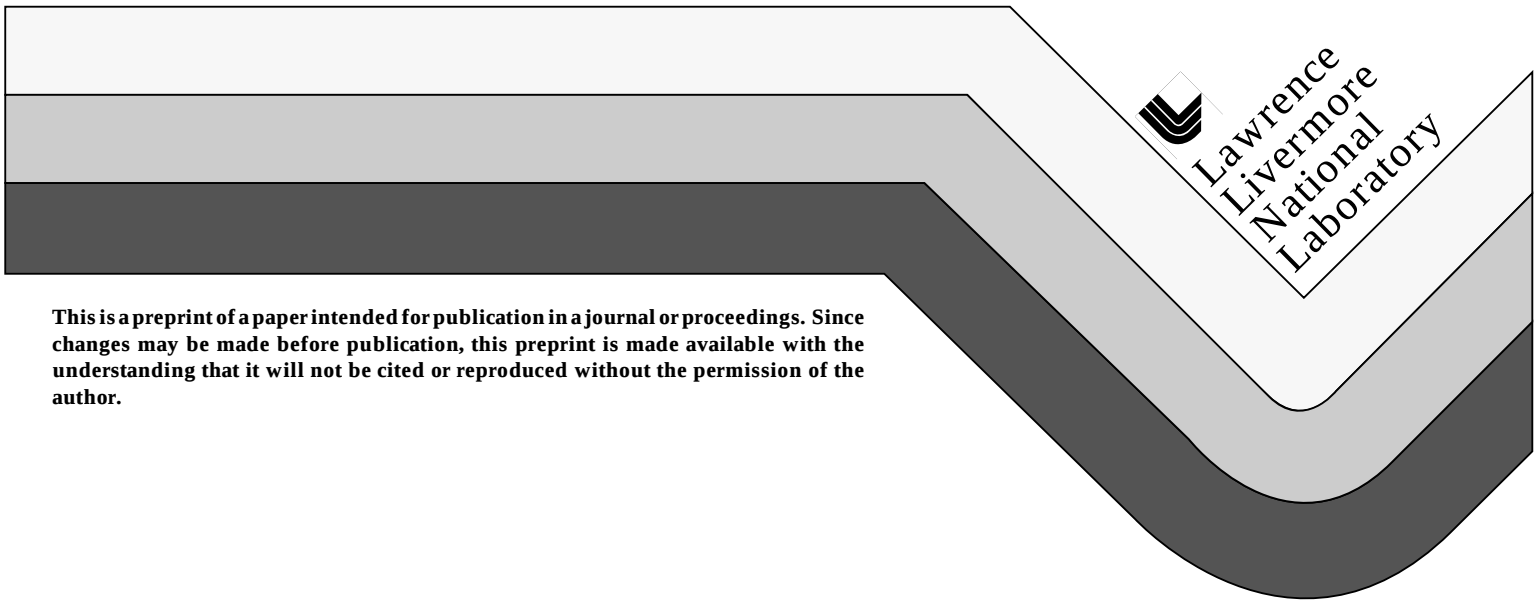

# Human calcium metabolism including bone resorption measured with $^{41}$Ca tracer

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Abstract

Accelerator mass spectrometry is so sensitive to small quantities of $^{41}$Ca that it might be used as a tracer in the study of human calcium kinetics to generate unique kinds of data. In contrast with the use of other Ca isotopic tracers, $^{41}$Ca tracer can be so administered that the tracer movements between the various body pools achieve a quasi steady state. Resorbing bone may thus be directly measured. We have tested such a protocol against a conventional stable isotope experiment with good agreement.

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## Background

Living bone constantly turns over calcium, i.e. calcium is both continually being absorbed and resorbed by bone as it reorganizes. This serves two purposes. Firstly, growing bone can reshapen to maintain the optimal form for support and then later mature bone can remodel itself in response to the strains upon it. Secondly, bone is also a reservoir of calcium acting to maintain calcium homeostasis; although nearly 99% of a body's approximately 1 kg of Ca is stored in the skeleton, the concentrations of the less than 1% available in soluble form in the extra- and intracellular compartments are crucial to cell function. Calcium movements within the body are shown schematically in Fig. 1. Ca absorption and resorption are usually closely coupled, but prolonged periods of even a small imbalance in favor of the latter reduces bone mass and weakens the bone. Eventually, typically late in life following the menopause in women, even minor trauma can cause the bone to fracture as a consequence. The study of this decline, so that one might potentially develop strategies to prevent, slow, halt or even reverse the bone loss and ultimately combat osteoporosis, requires measures of calcium kinetics in general and of bone resorption in particular. Great efforts to develop the means to generate ever more useful indices of resorption have been and will doubtless continue to be made as osteoporosis is a major public health problem that presumably will worsen as world populations age. Already in the US alone there are in excess of a million osteoporotic fractures a year with an associated financial cost of over $10 billion annually.

Bone resorption can be measured in several ways. The simplest approach in principle is to ascribe any measured imbalance between dietary and excreted calcium to the net movement of Ca into/out of bone. However, the amounts of calcium consumed and excreted are significantly larger than their difference such that these mass balance

measurements can be subject to large errors. In any case, mass balance studies are expensive requiring that the subjects remain in a metabolic ward so that diet can be controlled and all excreta collected. An alternate microscopic approach is the histomorphometry of bone biopsies. While this remains the only way to study bone at a tissue or cellular level, it is undesirably invasive and samples only a fraction of the skeleton. Precise measurements of single bone to whole skeleton mineral density are possible by X-ray absorptiometry. Such measurements separated in time can reveal changes to the bone. Absorptiometry is widely employed in research and for the clinical assessment of risk or diagnosis of osteoporosis, but other techniques are more suitable for quantifying or following variations in resorption. When the latter is the goal, one or more of a number of biomarkers can to used to monitor bone resorption rates. Blood and urine can be checked for evidence of broken-down collagen, some of which originated in bone, or the enzymatic activity of the osteoclast bone cells that do the resorbing. However, these indices can be too 'noisy' to reveal subtle effects because neither is specific enough to resorbing bone.

The remaining approach is to use calcium tracer enriched or made deficient in an isotope, either radioactive or stable. However, the very natures of the isotopes restrict their use protocols. Essentially, it is not always possible to administer enough tracer such that the tracer signal-to-noise is sufficient in the human derived samples. For instance, typically isotopic tracer does not permit a direct measurement of resorbing calcium. Such a measurement requires that tracer movements between the various body pools first approach a steady state reflecting the total calcium movements, rather than being driven by gradients in tracer concentrations between pools, and that the signature of resorption then be measurable. As only a small fraction of the skeleton resorbs in a day and that the amount resorbed is small compared to the greater rapidly cycling pool in which the

resorbing calcium must be detected, an at best impratically large amount of conventional isotopic tracer must be administered. Instead, the body calcium system is usually pulsed by the administration of one or more tracers and the transient response of the calcium circulation is measured. Following tracer administration *per os* or intravenously there is initially a net movement of tracer from the tracer rich blood to tracer poor bone, a situation that is then reversed as tracer free calcium from diet first displaces the tracer in the blood. At best one can follow the tracer time course, either directly in serum or indirectly in excreta, for the days that tracer remains detectable. Then, by applying a model of the circulation scheme, it is possible to derive values for bone calcium resorption with whatever confidence one has in that scheme. Potentially better, more direct, more model independent data can be generated if steady state tracer concentrations are attainable.

## $^{41}$Ca as tracer for human calcium kinetics study

Radioactive $^{41}$Ca is markedly different from any other isotope of calcium. $^{41}$Ca is negligibly abundant in nature, has a halflife of $10^5$ years and decays with the emission of soft X-rays that a dose of tracer is radiologically benign. In addition, $^{41}$Ca can be made plentifully by neutron activation of the major calcium isotope. In essence $^{41}$Ca combines the various advantages of the other isotopic tracers but without the associated disadvantages. It might therefore be added to the cannon of tracer isotopes now that accelerator mass spectrometry (AMS) has been shown to be able to detect such tracer. AMS is so sensitive to tiny quantities of $^{41}$Ca that a small amount of $^{41}$Ca tracer equates to a much greater quantity of any other. Indeed, the measurement is not even very challenging since the $^{41}$Ca concentration in calcium samples from human tracer studies can be many orders of

magnitude greater than are the levels to be found in samples for geochronology research, for which the AMS determination of $^{41}$Ca was originally developed. Also, instead of having to perform sample preparation chemistry that involves metalizing the Ca, simple wet chemistry is sufficient [1]. It is even possible that smaller accelerators than have hitherto been required for $^{41}$Ca AMS could suffice for biomedical measurements.

$^{41}$Ca tracer might variously be employed when other isotopes are precluded for physiological, radiological or economical reasons, including experiments involving tracer flows attaining a quasi steady state. This may be done in two ways. Most intuitively, bone might be labeled. In the days following the administration of tracer, the blood and extracellular tracer concentration, as also reflected in the excreta tracer levels, will initially fall as the part of the tracer bolus that is not absorbed by stable bone is naturally excreted from the body. Eventually, however, the extracellular tracer concentration will cease declining and plateau when the $^{41}$Ca level is maintained by the small amount of tracer resorbing from bone. At that time the measured level reflects bone calcium resorption. Another group's AMS measurements with an initial subject suggest that the period required until the plateau is attained is roughly 100 days [2]. However, an earlier elegant if unwieldy study in which subjects were confined to a metabolic ward and consumed a diet made deficient in the naturally occurring calcium isotope $^{48}$Ca, such that that remaining in the bone became a natural tracer for resorption, indicates that the plateau might be achievable, and subsequent experiments enabled, much more quickly [3]. We are further investigating this delay.

The alternative to labeling bone to measure resorption is to label everything except bone with $^{41}$Ca so that resorbing calcium can be identified by its lack of the isotope. This is done by continually administering tracer, or at least administering it frequently. The extracellular tracer concentration will then rise until it achieves a plateau level that depends

on the amount of relatively tracer free resorbing calcium. An older experiment of this type with radiotracer shows that this plateau can be achieved in approximately 10 days [4], a delay that might be reduced by initially priming the subjects with large amounts of tracer to rapidly bring their extracellular tracer levels close to the equilibrium condition.

The two protocols for achieving approaching steady state tracer flows might be employed in several ways. Each alone might provide a qualitative index of resorption much as the conventional biomarkers do. In this manner variations in resorption might be studied, for instance as a subject is challenged endocinologically or by diet. An intriguing possibility is the administration of $^{41}$Ca tracer to large populations as this is relatively inexpensive and the signals of resorption will remain lifelong. Subsequently, as is deemed appropriate, individuals' indices of resorption can be generated and examined. Alternatively, the protocols might be combined with other measurements, including each other, to generate various quantitative parameters of calcium kinetics including measures of bone calcium resorption and compete with mass balance and conventional tracer analyses. However, as this paper reports the successful detection of $^{41}$Ca tracer in only the second subject to whom it was administered, confirming that sample preparation and AMS detection techniques have been sufficiently developed, the exact utility of tracer needs to be better established before $^{41}$Ca tracer can be usefully employed in research or clinical practice. To this end we are attempting to compare $^{41}$Ca tracer derived data with mass balance, conventional isotopic tracer and biomarker derived results in on-going various collaborations with an additional 15 subjects.

A continuous feeding experiment

We have performed an experiment combining the two $^{41}$Ca tracer protocol types to demonstrate such and to establish a tracer dosimetry. A two week continuous feeding protocol was followed by measurements of resorbing tracer in a potentially powerful manner. A parallel conventional isotope test was also performed for comparison.

One 58 kg, 53 year old surgically menopausal female subject ingested 35 nCi $^{41}$Ca. The consumed tracer was in a total of 0.98 g $^{41}$Ca enriched calcium carbonate that was dissolved in calcium fortified orange juice and divided between three meals a day for two weeks. The tracer material was originally $^{40}CaCO_3$ from Oak Ridge National Laboratory that had received a thermal neutron fluence sufficient to transmute one in a million $^{40}$Ca atoms to $^{41}$Ca, subject to a 10% uncertainty in the neutron capture cross-section. A calculated dosimetry anticipated a radiological dose of 0.03 mrem/yr, a tiny fraction of the natural annual dose. Nevertheless, prior approval was obtained from the various institutional experimental review boards. A dose of 2.25 g/day polyethylene glycol (PEG) fecal marker was also consumed with the tracer. Occasional urine and fecal samples were taken and prepared as the fluoride salt for AMS measurement [1]. The results of the analyses are plotted in Fig. 2. For the final week, half a year after beginning the experiment, the subject returned to the original but now tracer free initial two week diet so that the calcium kinetics might be returned to the same state. On day 10 of the study, a standard dual stable isotope test was performed. That consisted of administering 8.7 mg $^{44}$Ca PO and 9.7 mg $^{42}$Ca IV, and of seven blood draws totaling 50 mL and a 24 hour urine collection in three 8 hour pools so that the rapid transport of these tracers through the body might be studied by thermal ionization mass spectrometry of these samples.

Consider the initial continuous feeding protocol. The blood and urine $^{41}Ca$ concentrations equilibrate at a plateau F where $F = \alpha \cdot {}^{41}Ca_{daily\ dose}/V_t$ and $\alpha$ is fractional absorption, $V_t$ is the daily Ca turnover rate and $^{41}Ca_{daily\ dose}$ is the known rate at which tracer is consumed. (This assumes that endogenously excreted Ca does not mix well with dietary Ca.) Knowing $\alpha$ (discussed below) and by using AMS to measure F, $V_t$ can be calculated. At the same time that there is the plateau F, there is a similar $^{41}Ca$ concentration F' in the feces. $V_F \cdot F' = (1-\alpha) \cdot {}^{41}Ca_{daily\ dose} + F \cdot V_{endo}$ where $V_F$ is total fecal Ca and $V_{endo}$ is the net endogenously excreted calcium. Later, following cessation of $^{41}Ca$ consumption, the urine and fecal $^{41}Ca$ concentrations decline towards levels E and E' where $E'/E = V_{endo}/V_F$. By using AMS to also measure F', E and E' it is possible to calculate the daily dietary amount of total Ca, $V_{in}$, daily fecal Ca and bone resorption, $V_{0-}$, which is the difference between turnover and absorbed dietary calcium (i.e. $V_{0-} = V_t - \alpha \cdot V_{in}$). That is to say, it is in principle possible to calculate all of the kinetics parameters with the exception of bone accretion, $V_{0+}$, and urinary Ca, $V_u$ (the latter of which can be relatively easily measured enabling calculation of the former), using only one isotope and only occasional urine and fecal sampling. Moreover, the values obtained are averages over the course of the experiment and are therefore potentially more meaningful than are the equivalents generated by conventional 'snap-shot' isotope studies.

However, this approach requires some assumptions. It assumes that the subject's Ca kinematics do not drift over the course of the study, but vary only a little about their averages. In this regard, the subject attempted to consume the same diet for the important beginning and ending measurements. Secondly, the above assumes knowledge of the averaged fractional calcium absorption. In principle this might be obtained by the use of a non-absorbed Ca analogue fecal marker. We optimistically attempted to use the PEG

consumed to this end, but were unable to sensibly normalize the total calcium in a sampled stool to the assumed complete daily fecal calcium using the measured stool PEG content. Instead we adopted the value of fractional absorption derived by the simultaneous but 'not averaging' stable isotope study. For a discussion of other more generic assumptions see reference [3].

In reality, there is not a single bone calcium pool but rather a series of pools with different characteristic times with which they turn over calcium. The stable isotope data was modeled with three pools: a 1 hour pool consisting of a notional rapidly turning over bone surface, the extracellular fluid and blood; a 1-2 day pool; and a 3-7 days pool considered to be deep bone. Longer pools are not accessible to experimentation with stable isotopes. The $^{41}$Ca steady state protocol was able to simply ignore these pools, considering any pool of less than 10 days as part of the single rapidly exchangeable pool including blood and the extracellular fluid. The protocol derived the rate at which calcium was transported through this pool and the rate of dietary input into the pool, and therefore, by difference, the amount of calcium resorbing from deeper pools. Since tracer administered continually does appear to plateau in the rapidly exchangeable pool after 10 days [4], it seems that a division of bone into a 10 days and faster pool, and longer term pools, is not inappropriate. In any case, defining resorbed calcium as calcium from pools of longer than 10 days is presumably more relevant to the study of long term Ca kinetics that affect osteoporosis than are the pools accessible to stable tracer use. Nevertheless, the transient stable isotope test and the steady state $^{41}$Ca protocol generated comparable results. Presumably in reality only a quasi steady state 10 days pool tracer concentration was achieved by continuous feeding, because tracer was increasingly returned from the longer bone pools with time. Likewise, after tracer consumption has ceased, the 10 days or less pool tracer concentration again only ever achieves a quasi steady state. This is because the

amount of tracer in the deeper pools from which the $^{41}Ca$ returns to the 10 days pool slowly diminishes with time as tracer is returned to that pool or else moves into ever deeper bone. The protocol is insensitive to this though. Presumably the change in rate of tracer resorption from the deeper bone over the time it takes resorbed calcium to mix in the faster pool is small after half a year of tracer resorption, as was the case here.

The parameters of calcium kinetics derived with the two types of calcium isotope experiment are compared in Table 1. In both cases the 9% value of fractional absorption, $\alpha$, generated with the stable tracers was used. This number was obtained by measuring the fraction of the dose of each of the stable tracers recovered in the urine. Similarly, measurement of the IV tracer in stool enabled stable tracer derived calculation of endogenous excretion. By modeling the tracer excretion data, estimating the subject's dietary calcium at the time of the stable isotope test and using the single day's measure of urinary calcium, it was possible to calculate the bone kinetics using the stable isotopes. The degree of consensus between the two very different approaches suggests that at the time of the measurement that the subject was losing about 100 mg of calcium from bone per day and was absorbing little to replace it. Clearly this state of affairs cannot continue long so that perhaps at the time the subject's kinetics were in a process of long term cycling about a healthier average state.

In conclusion, a continuous feeding experiment in which equilibration plateaus were assumed to have been achieved in urine and feces, generated data that agreed with results derived conventionally as best one could. That is, sampling and control of the subject's calcium kinetics were limited by the subject's normal, everyday lifestyle. The agreement suggests that steady state kinetics data can indeed be generated with $^{41}Ca$ tracer, something that should now be more thoroughly and properly tested, as we are attempting to do.

Acknowledgment

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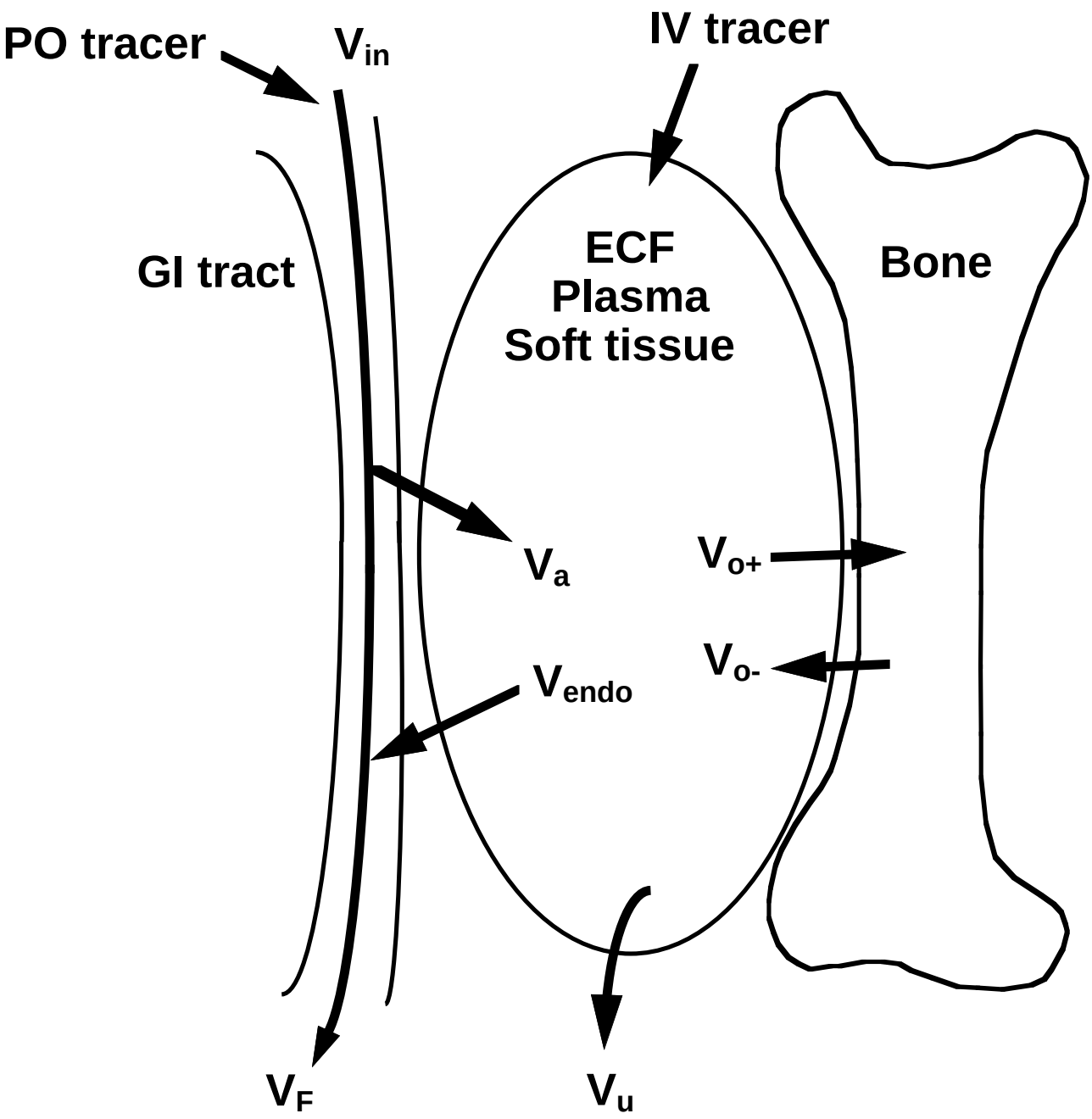
Figures

Figure 1. A schematic of simplified human calcium kinetics. The majority of dietary calcium, $V_{in}$, is rapidly passed from the body unabsorbed. A smaller amount, $V_a$, or $\alpha \cdot V_{in}$ where $\alpha$ is the so-called fractional absorption, is taken up from the gastrointestinal track and circulates in the extracellular fluid and blood. The endogenous fecal calcium, $V_{endo}$, is the part of this pool that makes a minor contribution to the total amount of calcium lost to feces, $V_F$. Another amount $V_u$ is passed in urine. $V_{0+}$ and $V_{0-}$ are the calcium flows to and from bone respectively. $V_t = V_a + V_{0-} = V_{endo} + V_u + V_{0+}$ is the rate of calcium turnover in the intermediate pool.

Figure 2. AMS measurements of the tracer concentrations in urine and fecal samples during and after a period of continuous tracer consumption. As data is insufficient to show the plateaus their existence is assumed. Note that although dietary calcium was similar after 160 days to that of the first two weeks, dietary calcium was less in the intervening period. Therefore, the second plateau conditions are more quickly and more steadily achieved than this graph suggests.

Table 1. Parameters of calcium kinetics generated with the $^{41}$Ca protocol and a conventional dual stable isotope test. The percentage errors are propagations of measurement uncertainties. Uncertainties in the amount of tracer administered are ignored as better characterized stock material is available. Avoidable uncertainties in the dual isotope test are similarly discounted.

| | | Stable isotope protocol | $^{41}$Ca protocol |
|---|---|---|---|
| | $\alpha$ | 0.09±<1% | use stable isotope value |
| Calcium transport rates (mg/day) | $V_t$ | 209±15% | 199±5% |
| | $V_u$ | 167±5% measured | |
| | $V_{endo}$ | 78±5% | 130±15% |
| | $V_F$ | | 1163±5% |
| | $V_{in}$ | 1200±10% estimated | 1135±5% |
| | $V_{o-}$ | 101±20% | 97±15% |
| | $V_{o+}$ | -36±15% | |


PO tracer
$V_{in}$
IV tracer
GI tract
ECF
Plasma
Soft tissue
Bone
$V_a$
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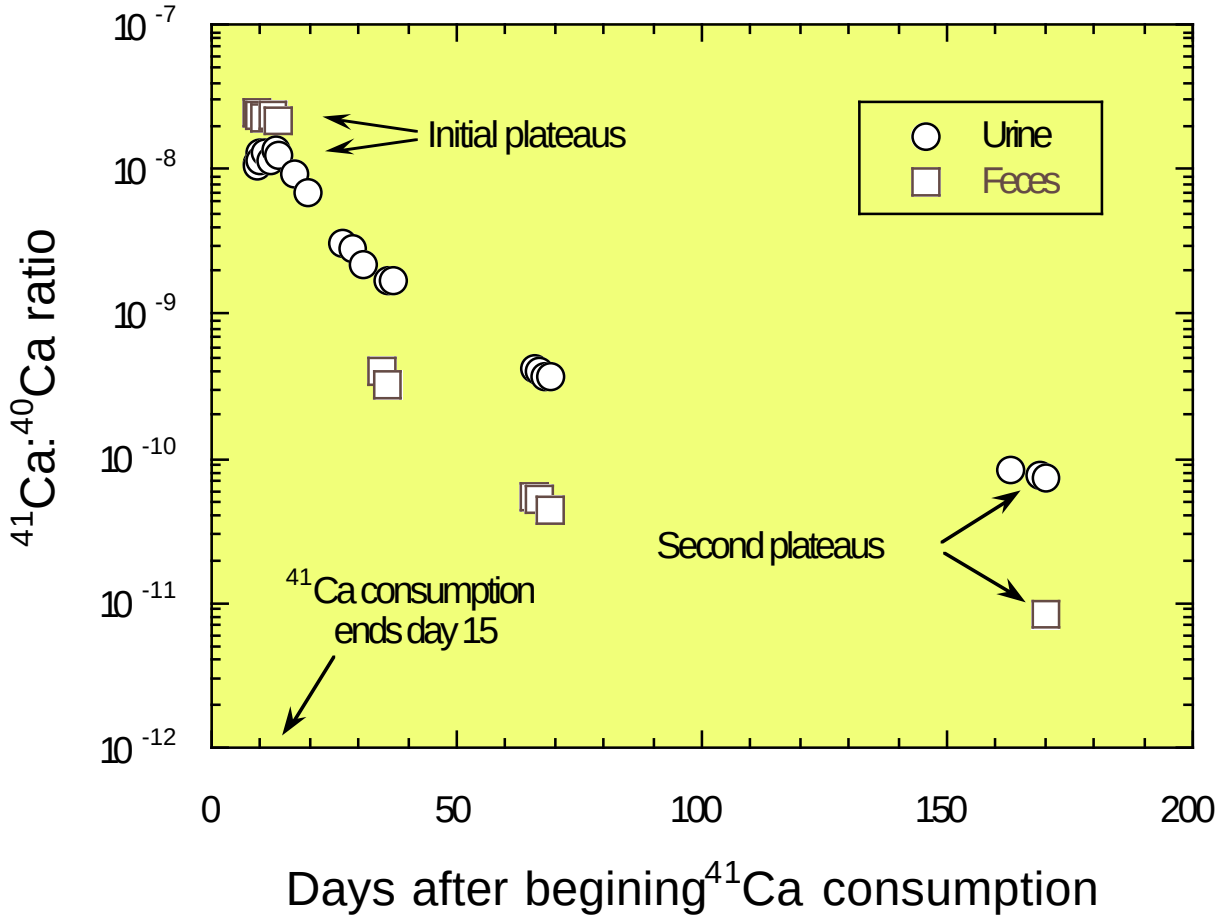
$10^{-7}$
$10^{-8}$
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$^{41}$Ca:$^{40}$Ca ratio
Initial plateaus
Urine
Feces
Second plateaus
$^{41}$Ca consumption
ends day 15
0
50
100
150
200
Days after begining $^{41}$Ca consumption